Cell Tissue Res (1999) 295:437-452

REGULAR ARTICLE

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Rapid anterograde and retrograde tracing from mesenteric nerve trunks to the guinea-pig small intestine in vitro

Received: 2 June 1998 / Accepted: 22 September 1998

Abstract A novel technique for rapid anterograde labelling of cut axons in vitro was used to visualise the peripheral branches of mesenteric nerve trunks supplying the guinea-pig small intestine. Biotinamide, dissolved in an artificial intracellular solution, was applied to the cut ends of the mesenteric nerves and the tissue was maintained in organ culture overnight. Labelled nerve fibres were visualised by fluorescein isothiocyanate (FITC)-conjugated streptavidin. Intense staining of nerve fibres and terminal varicosities in the ganglia and internodal strands of the myenteric plexus was achieved up to 15 mm from the application site. Filled fibres formed baskets around some myenteric nerve cell bodies, suggesting target-specific neurotransmission. When combined with multiple-labelling immunohistochemistry for tyrosine hydroxylase (TH), calcitonin gene-related protein (CGRP) or choline acetyltransferase (ChAT), most anterogradely labelled nerve fibres, and many pericellular baskets, were found to be TH immunoreactive, indicating their postganglionic sympathetic origin. Double-labelling immunohistochemistry revealed that the postganglionic sympathetic pericellular baskets preferentially surrounded 5-hydroxytryptamine (5-HT)-handling myenteric neurons. Some biotinamide-filled fibres were CGRP immunoreactive, and are likely to originate from spinal sensory neurons. We describe for the first time many pericellular baskets labelled from the mesenteric nerves which were ChAT immunoreactive. Retrogradely filled intestinofugal nerve cell bodies were also observed, all of which had a single axon arising from a small nerve cell body with short filamentous or lamellar dendrites. Many of these cells were ChAT immunoreactive. This in vitro technique is effective in identifying the fine arrangement of nerve terminals arising from nerve trunks in the periphery.

Key words Neural tracing · Biotinamide · Cholinergic neurons · Histochemistry · Visceral sensory neurons · Sympathetic neurons · Guinea-pig

Introduction

Determining the projections of individual classes of neurons is essential to understand how a neural system works. Neuroanatomists have employed two fundamental strategies to establish neural projections. One is based on lesion techniques, which lead to the degeneration of the nerve terminals disconnected from their nerve cell bodies (Jonsson 1981). More recently axonal tracing techniques have been used, based on either retrograde or anterograde axonal transport of exogenous substances (Groenewegen and Wouterlood 1990).

Anterograde tracing has been extensively used to determine the field of innervation of peripheral neurons by visualising the terminal axonal branching. This approach has been applied mainly to studies on whole animals with axons still connected to their nerve cell bodies (Aldskogius et al. 1986; Clerc and Condamin 1987; Neuhuber 1987; Lindh et al. 1989; Berthoud et al. 1991; Elfvin et al. 1992; Kressel et al. 1994), or less extensively in preparations in vitro with intact neurons (Anderson et al. 1978; Brookes et al. 1991, 1996). The tracing substance is usually applied to the nerve cell bodies or non-terminal axons, where it is taken up and transported to the nerve terminals. It would be useful to obtain anterograde labelling of severed axons in isolated preparations to study in detail the peripheral branching pattern of the afferent and efferent innervation of

This work was funded by Astra Pharmaceuticals. B.C.T. was in receipt of a Flinders 2000 summer scholarship, the CAMDO/AMSA undergraduate research fellowship, and the AMA J.G. Hunter Research Fellowship. S.J.H.B. was supported, for part of this study, by a Senior Research Fellowship in Digestive Science of the Gastroenterological Society of Australia.

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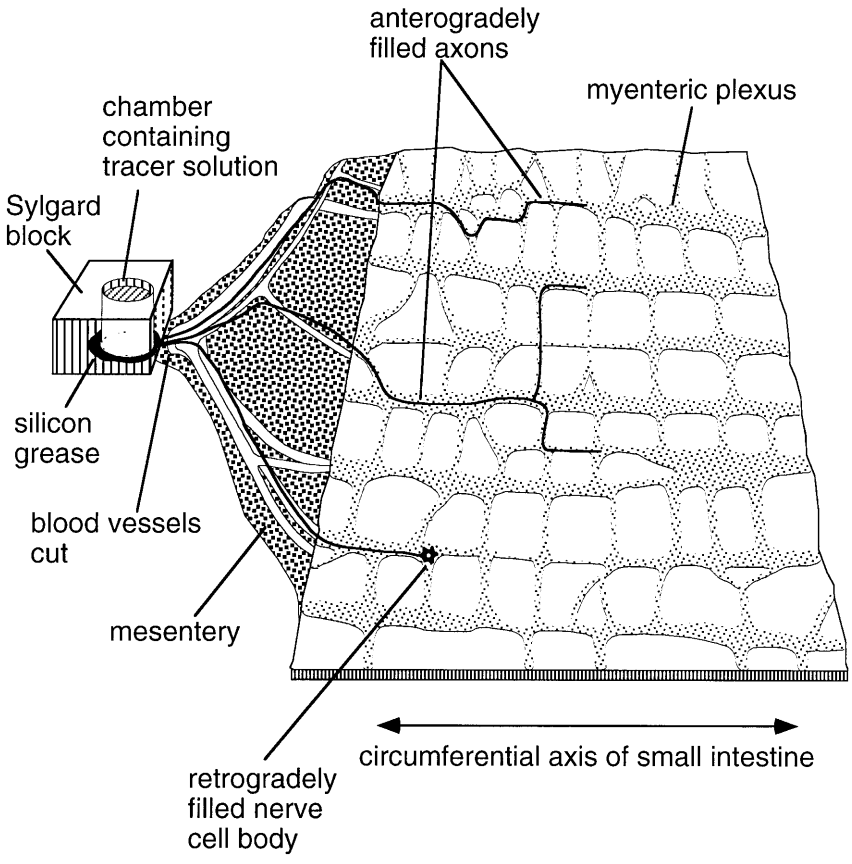

Fig. 1 Experimental set-up for filling nerve fibres in the mesenteric nerves. Biotinamide (2%) was applied to the cut end of the nerve in an artificial intracellular medium which was confined to a chamber made from a small block of Sylgard. This was sealed with a bead of silicon high-vacuum grease. The figure shows schematically anterogradely filled fibres (i.e. of extrinsic origin) and a retrogradely filled myenteric neuron (i.e. an intestinofugal neuron) in the myenteric plexus. For ease of display the mucosa and circular muscle layers are not represented

viscera. Such a technique should fill axons rapidly, as they degenerate after cutting (Williams and Hall 1971).

The intestine is supplied by extrinsic nerve fibres in the mesenteric nerves, both afferent and efferent. These include noradrenergic postganglionic sympathetic nerve fibres, as demonstrated by catecholamine fluorescence or immunohistochemistry for tyrosine hydroxylase (TH; Furness et al. 1979), a population of spinal afferent nerve fibres demonstrable with immunohistochemistry for calcitonin gene-related peptide (CGRP; Gibbins et al. 1987), preganglionic vagal parasympathetic nerve fibres, which have been demonstrated in anterograde labelling studies in vivo (Berthoud et al. 1991), and possibly vagal afferent nerve fibres (Eastwood et al. 1995). The branching patterns and terminals of many of these fibres remain largely unknown. It is known that a proportion of noradrenergic postganglionic sympathetic nerve fibres form baskets around myenteric neurons (Llewellyn-Smith et al. 1981), but it is not known whether they are around specific classes of myenteric neurons.

In this work we have developed a simple method for anterograde tracing in vitro, and have investigated visceral afferent and autonomic efferent nerve fibres supplying the guinea-pig small intestine by applying water-soluble tracing substances to the peripheral stump of severed mesenteric nerves. We have also used double-labelling immunohistochemistry for TH and neurochemical labels specific to certain classes of myenteric neuron to determine the nature of the myenteric targets of these sympathetic fibres.

Materials and methods

Dissection

Adult guinea-pigs (200–500 g) of either sex were killed by cervical dislocation followed by exsanguination, with the approval of the institutional animal ethics committee. Segments of ileum, 20–30 mm long with readily identifiable mesenteric blood vessels and nerves, were removed under aseptic conditions and placed immediately into sterile Krebs solution [117 mM NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, 1.2 mM NaH_2PO_4, 2.5 mM $CaCl_2$, 10 mM glucose, bubbled with 95% O_2 and 5% CO_2, pH 7.4, containing 1 μM nicardipine (Sigma, St. Louis, MO) to decrease smooth muscle contractions]. These segments were then flushed with Krebs solution and cut open longitudinally adjacent to the mesenteric attachment, thus leaving intact the extrinsic nerve connections to one-half of the intestinal wall. The tissue was stretched and pinned flat, mucosal side upwards, in a 9-cm-diameter Petri dish lined with Sylgard (Dow Corning, Midland, MC). Nerve trunks were dissected free from the mesentery up to 10 mm away from the small intestine, and the end pinned firmly. Mesenteric blood vessels were interrupted to prevent dye leakage.

Tracer solutions

In order to achieve fast penetration of labels into the cut axons, a number of different solutions were tested. Those which were not successful are briefly mentioned in the "Results". We describe here the method which proved successful in achieving anterograde labelling of cut axons in vitro.

The successful tracer solution was based on an artificial intracellular solution used for electroporation of cells (El-Gamal et al. 1992) to which we added membrane-permeabilising agents. The artificial

Table 1 Antibodies used

Primary	Supplier	Conc.	Reference	Secondary	Supplier	Conc.
Rabbit anti-NCB	Burcher (NCB-CB11)	1:800	Costa et al. 1996	Donkey anti-rabbit FITC	Amersham (N1034)	1:50
Mouse anti-calbindin	Celio (D-28 K)	1:1000	Celio et al. 1990	Donkey anti-mouse CY3	Jackson (25512)	1:100
Rabbit anti-calretinin	Rogers (AB-6-C)	1:3000	Rogers 1989	Donkey anti-rabbit FITC	Amersham (N1034)	1:50
Sheep anti-ChAT	Chemicon (AB1582)	1:5000	Porter et al. 1997	Donkey anti-sheep CY3	Jackson (26898)	1:100
Goat anti-5-HT	Incstar (108072)	1:2000	Kawahito et al. 1994	Donkey anti-goat CY3	Jackson (26035)	1:400
Sheep anti-NOS	Emson (K205)	1:1000	Williamson et al. 1996	Donkey anti-sheep FITC	Jackson (28126)	1:100
Mouse anti-SOM	Brown (Soma8)	1:1000	Buchan et al. 1985	Donkey anti-mouse CY3	Jackson (25512)	1:100
Rabbit anti-VIP	Walsh (7913)	1:800	Furness et al. 1981	Donkey anti-rabbit FITC	Amersham (N1034)	1:50
Rabbit anti-TH	Thibault (THth)	1:800	Thibault et al. 1981	Donkey anti-rabbit FITC	Amersham (N1034)	1:50
Mouse anti-TH	Incstar (22941)	1:600	Keast 1992	Donkey anti-mouse CY3	Jackson (25512)	1:100

intracellular solution consisted of 150 mM monopotassium L-glutamic acid (Sigma), 7 mM magnesium chloride, 5 mM glucose, 1 mM ethylene glycol-bis-(β-aminoethyl ether) *N,N,N',N'*-tetraacetic acid (EGTA), 20 mM hydroxyeicosapentaenoic acid (HEPES) buffer (Sigma), 5 mM disodium adenosine-5-phosphate (ATP, Sigma), 0.02% saponin (from Quillaja Bark, Sigma), 1% dimethyl sulphoxide (DMSO) and the tracer being tested. Solutions with ranges of pH 7.0–7.5 were found to be equally effective. Variations of the artificial intracellular solution included: substitution of 150 mM potassium gluconate (Sigma) for the potassium glutamate; substitution of 150 mM sodium gluconate (Sigma) for the potassium glutamate; saponin-free solution; increased saponin concentration (0.5%); DMSO-free solution; addition of calcium chloride (3 mM); and lowering the concentration of potassium glutamate to 119 mM, making the tracer solution isotonic. Two percent biotinamide in culture medium was also tested as a tracer solution, and 80 μM colchicine (Sigma) was included in the culture medium of four preparations with biocytin tracer, and four with biotinamide tracer, to establish the role of microtubule-dependent axonal transport.

The tracers tested were: 2% Neurobiotin [*N*-(2-aminoethyl) biotinamide hydrochloride; Vector, Burlingame, CA] or *N*-(2-aminoethyl) biotinamide hydrobromide (Molecular Probes, Eugene, OR) (which are collectively referred to as "biotinamide" throughout this paper), 2% biocytin (Sigma), 0.5% biotinylated wheat-germ agglutinin (WGA) (Molecular Probes), 1% fast blue (Sigma), 2% fluorogold (Fluorochrome Inc., Eaglewood, CO), 0.5% miniruby (biotin dextran rhodamine, MW 10 000; Molecular Probes), and 0.5% microruby (biotin dextran rhodamine, MW 3000; Molecular Probes).

Tracer application and organ culture

A 10-mm-thick block of Sylgard, with a 5-mm-diameter hole bored through it, was used as a chamber for application of the tracer solution. Thin beads of high-vacuum silicon grease (Dow Corning) were applied, using a syringe fitted with a 26-gauge needle, both around the base of the hole in the Sylgard block and to the Sylgard underneath the mesenteric nerves. The Sylgard block was placed over the cut end of the mesenteric nerve, forming a watertight seal (Fig. 1). The Krebs solution contained within this chamber was removed, and 40–50 μl tracer solution applied. At 10-min intervals the chamber was again drained and fresh tracer solution applied for a total of three applications. The preparation outside the chamber was then rinsed 3 times with sterile Krebs solution, and 20 ml sterile culture medium was applied (DME/F12, Sigma) supplemented with 10% foetal bovine serum (v/v heat inactivated), 1.8 mM $CaCl_2$, 100 IU/ml penicillin, 100 μg/ml streptomycin, 2.5 μg/ml fungizone, 20 μg/ml gentamycin (Cytosystems, NSW, Australia) and adjusted to pH 7.4. The Petri dish was placed on a rocking tray (tipping at 0.8 Hz) in a humidified incubator in 5% CO_2 in air for 12–72 h at either 24°C or 37°C. Culture medium was changed daily (Brookes et al. 1991).

Tissue preparation

Specimens were fixed for 24 h at 4°C in modified Zamboni's fixative (15% saturated picric acid and 2% formaldehyde in a 0.1 M phosphate buffer, pH 7.0), then rinsed in phosphate-buffered saline (PBS; 150 mM NaCl in 100 mM sodium phosphate, pH 7.2; 3×10 min). The mu-

cosa and circular muscle layers were removed and preparations were permeabilised by washing 3×10 min in DMSO, or 2 h in 100% glycerol. After clearing, the preparations were washed 3×10 min in PBS.

Histochemistry

The biotinamide, biocytin, and biotinylated wheat-germ agglutinin were visualised using fluorescein isothiocyanate (FITC)-conjugated streptavidin (Amersham Life Sciences RPN1232, Sydney, NSW, Australia, 1:50, 2 h). Tissue was incubated in primary antisera for 24 h at room temperature. It was then rinsed 3 times in PBS and incubated in secondary antisera for 2 h. The antisera used in this study are listed in Table 1. The mesenteries were then removed to allow preparations to be mounted flat in bicarbonate-buffered glycerol (pH 8.6).

Microscopy

Fluorescently labelled preparations were viewed on an Olympus AX70 microscope fitted with epifluorescence with filters from Chroma Technology Co. (Brattleboro, VT). FITC was viewed using set 31001NB (excitation 470/90; dichroic 505; emission 515/45). CY3 was viewed using set 31002 (515/50; 565; 575/615), CY5 using set 41008 (590/650; 660; 665/740) and AMCA was viewed using set 31000 (340/80; 400; 435/85). This combination of filters ensured selective viewing of individual fluorophores. Images were captured via a Sony (MSC1708) black and white video camera and recorded on an Apple Power Macintosh 7100 computer using NIH Image 1.62 software (NIH, Bethesda, MA) via a Scion LG-3 frame grabber board (Scion Co., Frederick, MA).

Images obtained by video frame grabbing or confocal microscopy were cropped, scaled, brightness and contrast adjusted and fast Fourier transformations run (to remove the fine striations present in images with high contrast) in NIH Image 1.62. No other modification procedures were used unless specifically mentioned. Images were scaled, aligned and labelled in Microsoft Powerpoint 4 and printed on a Kodak XLS-8600 PS dye sublimation printer at 300 dots/inch.

The branching patterns of labelled axons were analysed using a computerised plotting system (Brookes et al. 1992), which recorded the microscope stage position on two linear scales with 1 μm resolution (Mitutoyo, Tokyo, Japan). Coordinates were collected by software developed specifically for this purpose and preparations were reconstructed using graph-plotting software (SigmaPlot, Jandel, Corte Madera, CA; Song et al. 1991). The sites of labelled fibres, pericellular baskets and intestinofugal cell bodies, and the outlines of the preparation, were recorded. A small proportion of well-labelled fibres were mapped in detail to reveal their branching pattern within the myenteric plexus.

Confocal microscopy

Laser scanning confocal microscopy was used to demonstrate the three-dimensional structure of selected anterogradely labelled pericellular baskets and retrogradely filled intestinofugal neurons. The cells and baskets were selected according to the quality of anterograde labelling, and suitability for three-dimensional reconstruction.

A Bio-Rad MRC-1024 combined with an Olympus AX70 microscope equipped with a krypton/argon laser with an excitation wavelength of 488 nm (for visualising FITC) was used. Optical sections were acquired using a 60× Olympus oil immersion lens (numerical aperture: 1.4) with the confocal iris set to 1–2 mm. The preparations were scanned at 0.5-μm steps to obtain a Z-series of up to 65 optical sections per basket or nerve cell body. This method enabled accurate resolution of varicosities within the baskets. The microscope was linked to a Compaq Pentium PC, running Laser Sharp software (Bio-Rad Microscience, Herts, UK) for instrument control and data acquisition.

Images were transferred to a Apple Macintosh Power PC 7500/100, and individually prepared for presentation in three ways using NIH Image version 1.62. The optical sections were condensed to a single image (z-projection) in order to provide a high-quality photograph, with all filled structures in focus. Alternatively, stereo pairs were created, with a depth disparity of 0–7 pixels.

For three-dimensional reconstruction of retrogradely labelled nerve cells, images were prepared for reconstruction using a thresholding technique to distinguish labelled structures from background fluorescence. They were then converted to one-bit (black and white), and median routines were used to reduce noise. The image files were then transferred to a Silicon Graphics Indigo 2 workstation (Mountain View, CA, USA) running Image Volumes 2.1 (Minnesota Data Corporation, MN) for three-dimensional reconstruction. Surfaces of cells and varicosities were generated using a marching cubes algorithm. Two iterations of smoothing were applied to the reconstructed objects to reduce contouring effects.

Immunohistochemical identification of myenteric targets of sympathetic nerve fibres

In order to determine whether a specific class of myenteric neuron was contained within the baskets of sympathetic postganglionic nerve fibres, double-labelling immunohistochemistry was used. Ten-centimetre segments of distal ileum were removed from nine adult guinea-pigs and placed immediately into PBS. The lumen of the ileum was flushed with PBS. After cutting open along the mesenteric border of the tissue, the segments of ileum were stretched and pinned flat, mucosal side upwards, in a 11-cm Sylgard-lined glass Petri dish.

Tissue was fixed, permeabilised using DMSO and dissected as described previously. Squares of tissue (5–10×5–10 mm) were then processed for double-labelling immunofluorescence, using primary antisera for TH and for neurochemical labels specific for different classes of myenteric neuron (Costa et al. 1996). To enable quantification of the proportion of enteric neurons surrounded by extrinsic nerve fibres, four preparations were processed for double-labelling immunofluorescence for TH and a label contained in all nerve cell bodies in the myenteric plexus, which is referred to as the nerve cell body (NCB) antiserum (Costa et al. 1996). Specimens were incubated with primary antibodies for 24 h, followed by secondary antibodies for 2 h. The antibodies used are shown in Table 1. Double labelling was performed with TH (Incstar) in combination with each of the following antisera: ChAT, NCB, nitric oxide synthase (NOS), and vasoactive intestinal polypeptide (VIP). Similarly the Thibault anti-TH antibody was combined with calbindin, calretinin, 5-hydroxytryptamine (5-HT) or somatostatin (SOM) antisera.

5-HT immunoreactivity in enteric neurons is very low in tissue fixed immediately after killing the animal, but after incubation with 5-HT the staining is more intense (Costa et al. 1982). Therefore tissue to be stained for 5-HT-handling neurons was treated differently. Segments of small intestine from four animals were placed into sterile Krebs solution and the lumen flushed. They were then cut open and pinned flat in Sylgard-lined glass Petri dishes. After the mucosa and submucosa were dissected off, the Krebs solution was removed and replaced with fresh Krebs solution containing 10^{-4} mM 5-HT (creatinine sulphate complex, Sigma). Tissue was incubated for 3 h at 37°C in a humidified incubator, then rinsed 3 times at 10-min intervals in PBS before fixation. Specimens were whole-mounted in bicarbonate-buffered glycerol and viewed as described previously.

Preparations were viewed systematically by scanning preparations in the *x*-axis at intervals of 50 μm. When a TH-immunoreactive pericellular basket was found, the immunoreactivity of the nerve cell body contained within was recorded. In preparations stained for NOS, VIP, calbindin, calretinin, SOM and 5-HT, 30–40 nerve cell bodies were chosen at random, and the immunoreactivity of that cell recorded.

In tissue stained for NCB and TH, ten myenteric ganglia were chosen randomly. The number of nerve cell bodies and the number of TH-immunoreactive baskets were counted to enable calculation of the number of cells which are surrounded by TH-IR baskets.

Data were prepared using Microsoft Excel 5.0 (Microsoft, Redmond, WA), and tests for statistical significance were performed using Statview II software (v1.03, Abacus Concepts Inc., Berkeley,

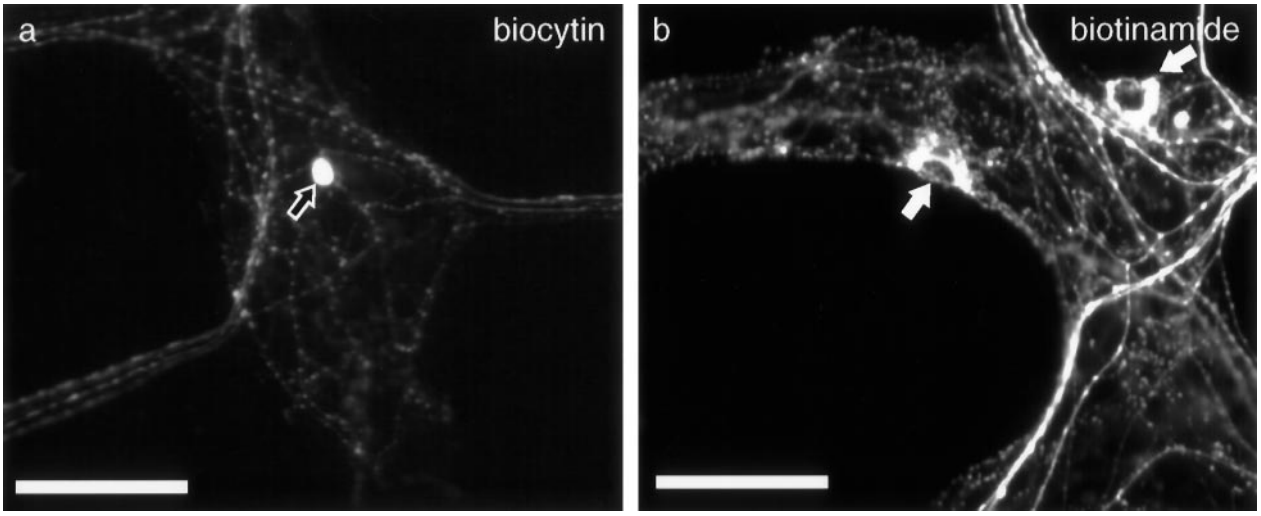

Fig. 2a,b Differences in quality of anterograde filling between biocytin and biotinamide. Biotinamide (**b**) anterogradely stained more fibres, more intensely, and for a longer distance than did biocytin (**a**). Two pericellular baskets can be seen in the biotinamide-filled preparation (*arrows*). The retrogradely filled nerve cell body of an intestinofugal neuron can be seen in the biocytin-labelled preparation (*unfilled arrow*). *Bar* 100 μm

CA). The proportion of cells within TH-immunoreactive baskets which contained the label being tested was compared to the proportion of randomly selected cells in the same preparation which contained that label using a two-tailed paired *t*-test. Results were presented as means±SEM.

Results

Initial tests with tracer solutions

Initial experiments showed that biocytin in either Krebs solution or standard culture medium applied to the cut ends of the mesenteric nerves rarely labelled axons. Crushing the nerves with biocytin-coated forceps produced variable labelling of axons in three of six preparations. Solutions of 2% biocytin or biotinamide in distilled water (hypotonic solution) also failed to label axons as did hypotonic solutions of 2% biocytin with 50 mM KCl to depolarise membranes. We considered that this may have been due to resealing of the axonal membranes after cutting. Therefore 1–10% ethanol, 10% glycerol or 10% DMSO was added to this tracer solution to permeabilise the membrane. Faintly labelled axons were seen within the mesentery using these solutions, but no labelled nerve fibres were found in the myenteric plexus of six preparations. ATP has previously been shown to permeabilise certain tumour cell-membranes (Hempling et al. 1969). Addition of 1 mM disodium ATP to a solution of 2% biocytin in 50 mM KCl did not significantly improve labelling in any of the six preparations tested. These results led us to use a full intracellular medium as the vehicle to deliver tracer to the axoplasm. This gave the most complete and reliable labelling (see below).

Artificial intracellular solution

Following these initial studies, a number of different tracers were then systematically tested in the artificial intracellular solution (El-Gamal et al. 1992). Intensely labelled varicose and non-varicose fibres were seen in the myenteric plexus after 20 h in organ culture using both biocytin and biotinamide (Fig. 2). Best labelling was consistently achieved using either Neurobiotin or *N*-(2-aminoethyl biotinamide) hydrobromide (Fig. 2b). The labelling with these two chemically similar tracers could not be distinguished and is hereafter referred to as “biotinamide”. Biocytin-labelled fibres were less intensely labelled and were not filled for as far as biotinamide-labelled fibres. No labelled fibres were seen using biotinylated wheat-germ agglutinin, fast blue, fluorogold, miniruby, or microruby under similar culture conditions.

Following applications of biotinamide, labelled fibres were seen in the mesenteric nerves, and in the ganglia and internodal strands of the myenteric plexus. Well-labelled smooth and varicose nerve fibres could be followed in the myenteric plexus, and numerous labelled varicosities were visible within the ganglia (Fig. 2b). A small number of fibres ran outside the internodal strands and ganglia for short distances.

A large number of fibres were labelled from a single application of tracer to mesenteric nerves, covering a large area of myenteric plexus (Fig. 3). Labelled varicose fibres formed a slender network within ganglia (e.g. Figs. 2b, 9b). In ganglia further from the tracer application site, some fibres clearly ended in varicosities (see Fig. 9b). A small number of thick fibres terminated in a coarse bulb. Labelled fibres ran predominantly circumferentially, and were approximately restricted to the half of the preparation which maintained connection to the mesenteric nerves; i.e. they

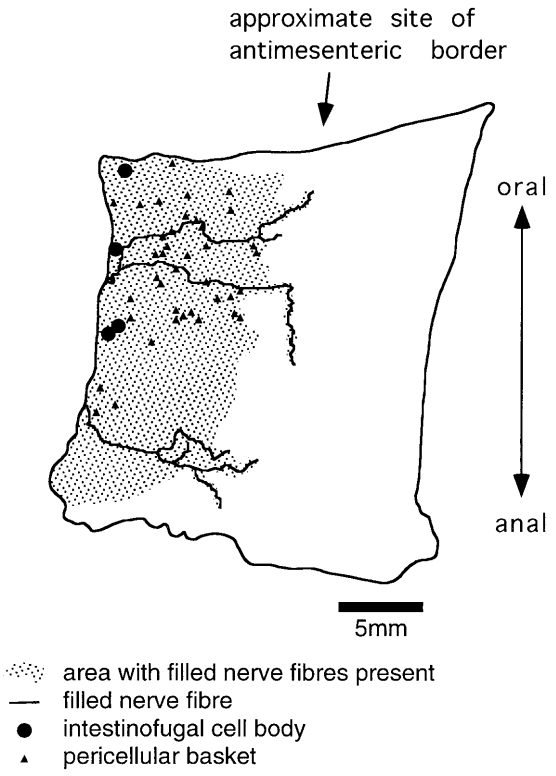

Fig. 3 Plot of the distribution of nerve fibres filled from the mesenteric nerves in one preparation. The mesenteries had to be removed prior to mapping in order to mount the preparation, but were originally attached to the left side of the preparation and the biotinamide was applied 3–5 mm from the edge of the preparation. Fibres were filled for over 15 mm from the application site on the mesenteric nerves (not shown). The full course of a few representative well-labelled fibres was plotted (*black lines*). The positions of pericellular baskets (*triangles*) and intestinofugal cell bodies (*circles*) are also shown. Note that filled fibres rarely crossed the antimesenteric border of the preparation (corresponding to the midline of the preparation)

did not project far beyond the antimesenteric border. Many of the fibres were seen to branch (Figs. 3, 4a).

Intensely labelled pericellular baskets consisting of fine varicose fibres and terminals were labelled in many myenteric ganglia (Figs. 2b, 3, 4b,c) and appeared to surround individual enteric nerve cell bodies which lacked detectable labelling. There were 20±6 baskets of filled nerve fibres per 20- to 30-mm-long preparation (n=4). Examples of typical baskets consisting of anterogradely labelled nerve fibres are shown in Fig. 4b,c.

Retrogradely labelled nerve cell bodies were also found in most preparations (Figs. 3, 5). They were usually located in myenteric ganglia close to the mesenteric border. These nerve cell bodies were round or oval in shape, with a single axon and a variable number of relatively short filamentous or lamellar dendritic processes. These cells were small, with average dimensions of 26.0±3.9×15.7±3.1 μm (19 cells). Three-dimensional reconstructions of such nerve cell bodies are shown in Fig. 5c,d. These are intestinofugal neurons, which are known to project to prevertebral ganglia (Dalsgaard and Elfvin 1982; Kuramoto and Furness 1989).

Modifications of tracing method

The artificial intracellular tracer solution described above gave the best filling of cut axons. The mechanisms by which tracers entered the cut axons and were transported are unclear. In order to establish which components of the tracer solution were critical for optimal filling, different components of the tracer solution were systematically altered.

Lowering the concentration of potassium glutamate to 118 mM (making the solution isotonic) produced an intensity and distance of labelling equivalent to that obtained with the standard tracer solution and no differences were detectable between solutions containing glutamate and gluconate as the major anions. However, substitution of sodium gluconate for potassium glutamate decreased the number and distance of fibres filled, and the intensity of staining. Addition of 3 mM calcium chloride to the artificial intracellular solution made filling considerably less consistent, with fewer fibres labelled (Fig. 6). In two out of five preparations, the few well-filled fibres could be followed as far as when labelled using standard tracer solution, but were less intensely stained. The remaining three preparations were filled for a shorter distance, and were stained less intensely than when standard tracer solution was used (Figs. 6, 7). The permeabilising agents, saponin and DMSO, were not essential for adequate filling, but they appeared to increase the intensity of staining slightly. When the artificial intracellular tracer solution was replaced with standard supplemented DME/F12 containing 2% biotinamide, a small number of fibres were faintly labelled for a short distance into the myenteric plexus.

Addition of 80 μM colchicine to the culture medium in the Petri dish for the duration of the culture only slightly reduced the distance of filling and intensity of staining (Fig. 8), suggesting that the anterograde labelling is predominantly independent of microtubule-dependent axonal transport. In order to further investigate the nature of the anterograde transport, the temperature and the duration of the incubation in culture were investigated. Following incubation at 24°C, adequate labelling was obtained, but it required a longer duration of culture (48–72 h) to achieve labelling distances and intensity of staining equivalent to that after 20 h at 37°C. Prolonging the culture period at 37°C beyond 20 h led to the appearance of signs of degeneration, with swollen and fragmented axons. Similarly, culturing the tissue for longer than 72 h at 24°C also led to signs of degeneration. These results indicate that the temperature and duration of incubation are important determinants of extent of anterograde labelling.

Combination of anterograde tracing and immunohistochemistry

Forty-three preparations anterogradely labelled under optimal incubation conditions (20 h at 37°C) were processed for immunoreactivity for TH and CGRP. This staining appeared similar to that in freshly fixed controls, suggesting

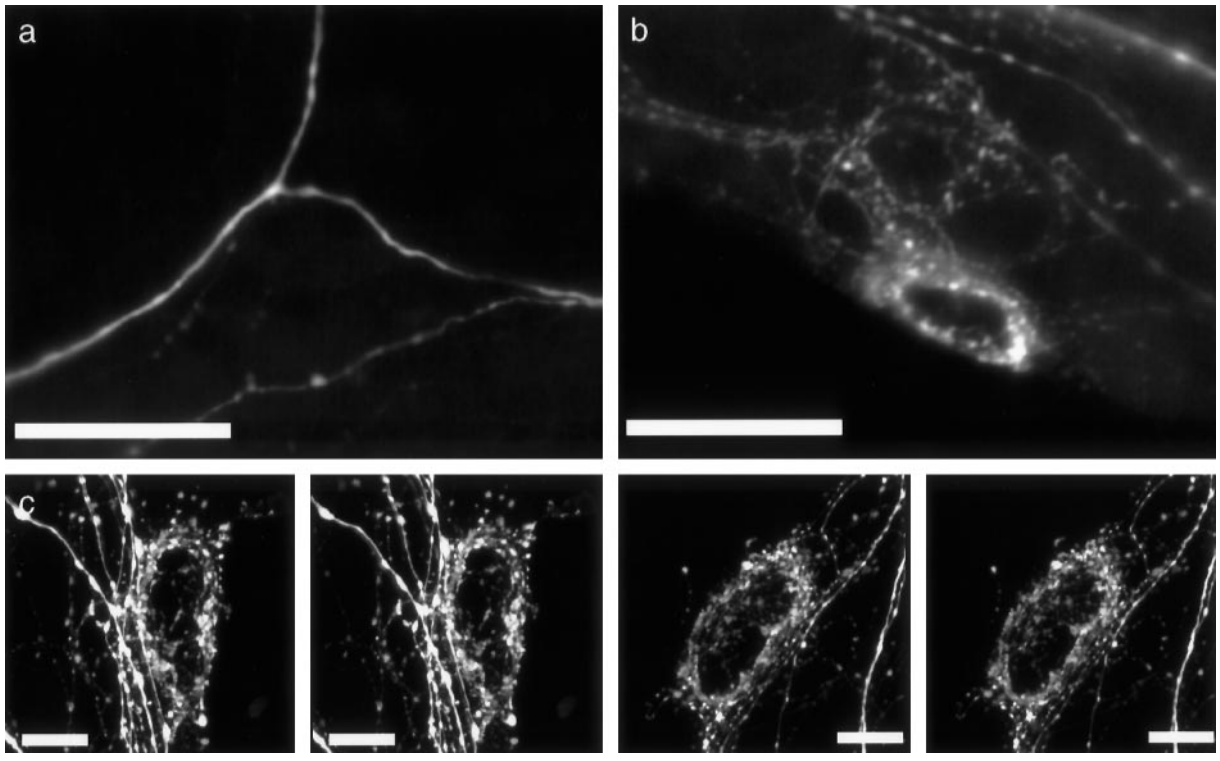

Fig. 4 **a** Several anterogradely filled axons, one with a branch point within a ganglion of the myenteric plexus. **b** Anterogradely filled nerve fibres forming a basket in a myenteric ganglion, surrounding a non-labelled cell body of a myenteric neuron. **c** Two stereo pairs of confocal micrographs of baskets of anterogradely labelled nerve fibres in the myenteric plexus. Note that the varicosities appear to form a ring around the perimeter of the unlabelled nerve cell body rather than enclosing it in a spherical arrangement of varicosities. *Bars* 50 μm (**a**), 50 μm (**b**), 20 μm (**c**)

that minimal degeneration of the severed axons had occurred. Many fibres in the ganglia and internodal strands of the myenteric plexus were immunoreactive for TH. CGRP immunoreactivity was present in only a few fibres (Fig. 9). No coexistence of TH and CGRP was observed, confirming previous results (Gibbins et al. 1987).

Most of the anterogradely labelled nerve fibres showed immunoreactivity for TH (Fig. 9). Anterograde labelling visualised the non-varicose and intervaricose parts of fibres more clearly than TH immunoreactivity (Fig. 9), allowing individual nerve fibres to be followed within the ganglion. Because only a small proportion of the TH-immunoreactive nerve fibres in ganglia which were not immediately adjacent to the mesenteric border were anterogradely labelled, the branching patterns of these fibres could be readily identified (Fig. 9b). A small number of anterogradely labelled fibres were immunoreactive for CGRP, and these appeared to branch less extensively than the TH-immunoreactive fibres (Fig. 9e). Only a few anterogradely labelled nerve fibres that were not immunoreactive for either TH or CGRP were seen (Fig. 9g–i). As such fibres were mixed with the larger number of labelled fibres immunoreactive for TH or CGRP, they were difficult to identify.

In the four anterogradely labelled preparations stained for TH and ChAT, all of the anterogradely labelled pericellular baskets of varicosities were analysed in detail: 64±12% contained some varicosities with TH immunoreactivity. In the remaining 36±12% of the labelled baskets, few if any of the varicosities showed detectable TH immunoreactivity (Fig. 10); 39±8% of the analysed baskets contained varicosities with ChAT immunoreactivity (Fig. 10e,f). However, due to the faint ChAT immunoreactivity, it is possible that a greater proportion of baskets actually contained cholinergic nerve fibres; 23±4% of anterogradely labelled baskets contained both varicosities with TH and varicosities with ChAT immunoreactivity, although no varicosities showed colocalisation of TH and ChAT immunoreactivities in any preparation. No labelled pericellular baskets contained varicosities immunoreactive for CGRP. Detectable ChAT immunoreactivity was present in 36±10% of the nerve cell bodies within anterogradely labelled baskets. However, we believe that this may be misleading, as ChAT labelling was optimised to allow visualisation of nerve terminals, making it likely that many of the remaining nerve cell bodies surrounded by baskets had ChAT immunoreactivity too faint to be detected against the high background labelling in ganglia (Steele et al. 1991).

There were 18 nerve cell bodies retrogradely filled in the four anterogradely labelled preparations stained for TH and ChAT (4.5±1 per 20- to 30-mm preparation). Of these, 20±14% were detectably immunoreactive for ChAT, although a greater proportion of these neurons may have levels of ChAT too low to be detected. As expected, the

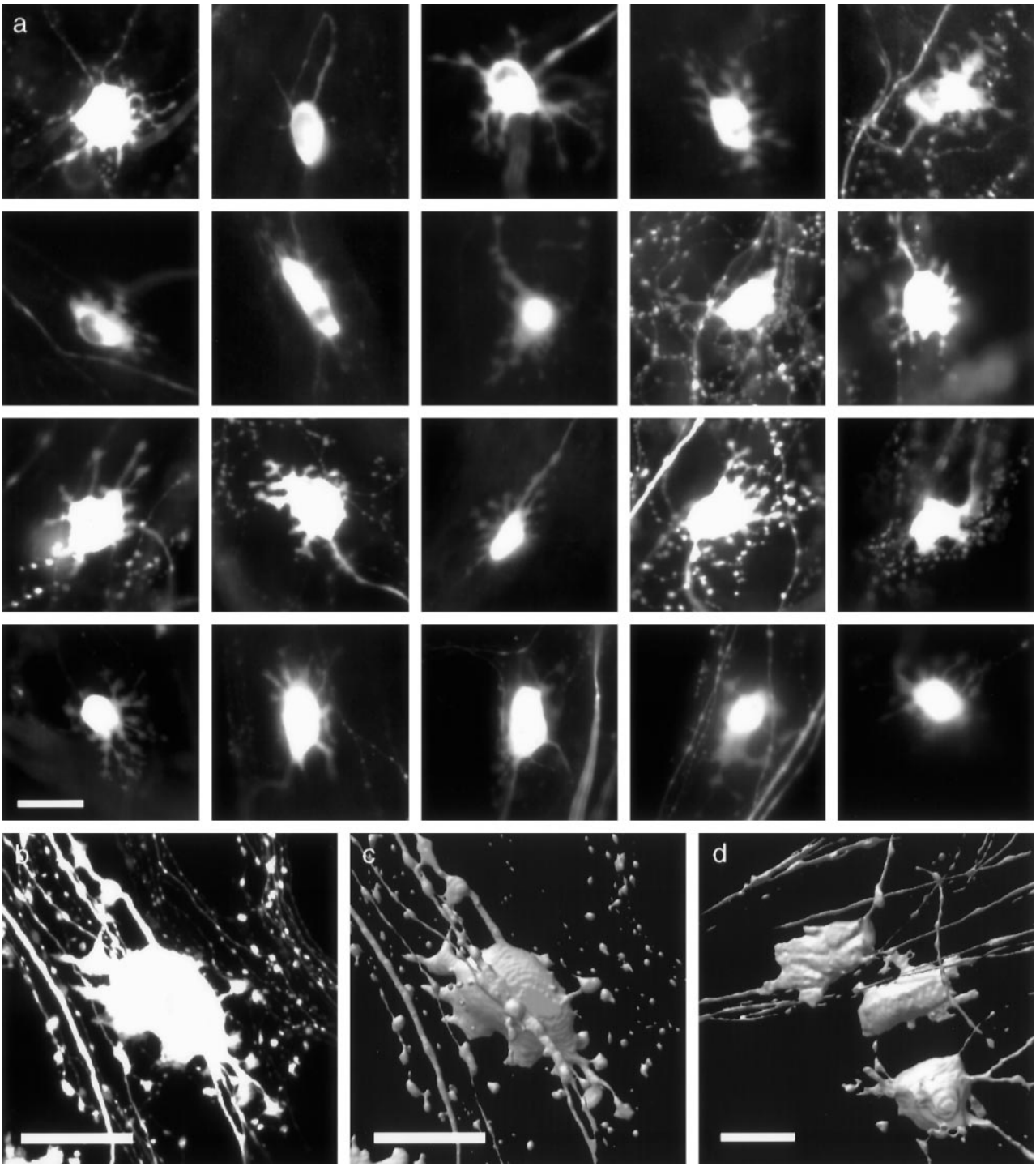

Fig. 5 **a** Nerve cell bodies of intestinofugal neurons retrogradely labelled from the mesenteric nerves. Note the diverse morphology of these neurons, although all appeared to have a single axon and lamellar and/or short filamentous dendrites. **b–d** Confocal micrographs of retrogradely labelled intestinofugal neurons. **b** Merged vertical projection of the optical sections. **c** Three-dimensional reconstruction of the same intestinofugal neuron. **d** Three-dimensional reconstruction of three of retrogradely labelled intestinofugal nerve cell bodies. *Bars* 30 μm (**a**), 20 μm (**b–d**)

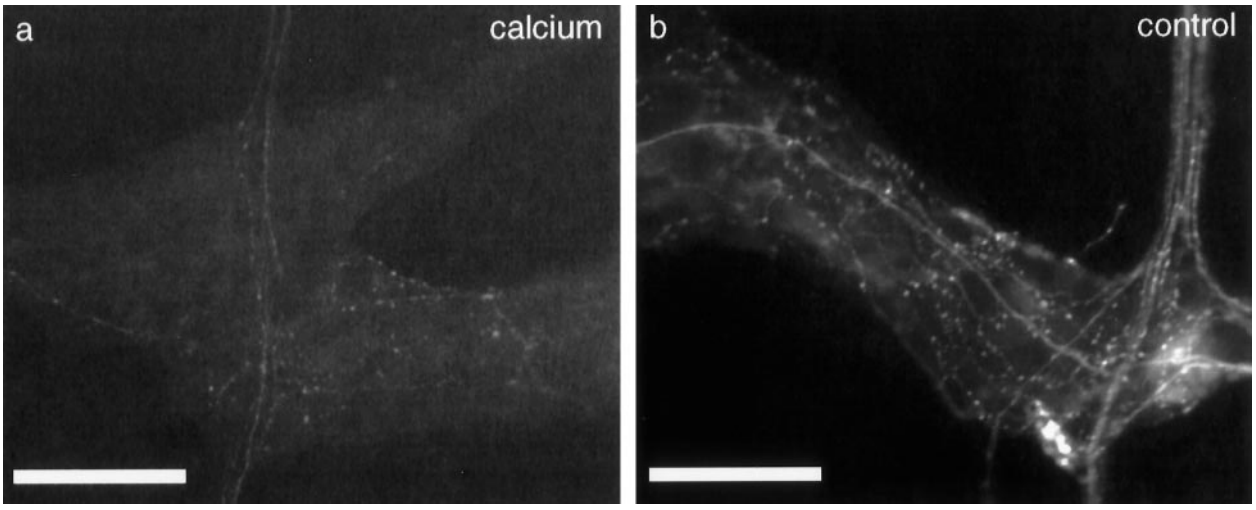

Fig. 6a,b Comparison of labelling between preparations with tracer solution containing 3 mM calcium chloride (**a**) and standard tracer solution (**b**). Tracer solution containing calcium chloride antero-gradely filled fewer fibres, and these only faintly, in comparison to the standard tracer solution. *Bar* 100 μm

intestinofugal neurons were not immunoreactive for either TH or CGRP.

TH-immunoreactive baskets and enteric neuronal markers

Both of the antibodies against TH provided good labelling of TH-immunoreactive fibres and varicosities. TH-immunoreactive pericellular baskets were observed in all preparations; 0.6±0.1% of cell bodies in the myenteric plexus were surrounded by a basket of TH-immunoreactive varicosities. No baskets of TH-immunoreactive varicosities were observed which were not around nerve cell bodies. The proportion of cells contained within TH-immunoreactive baskets which were immunoreactive for the various neuronal markers tested is shown in Table 2.

Immunohistochemistry for ChAT was more variable than for the other labels used. At least 61±5% of the nerve cell bodies surrounded by TH-immunoreactive baskets were immunoreactive for ChAT although, again, it is likely that this may be an underestimate due to the faint nature of ChAT staining, since it was frequently difficult to determine whether a cell body contained ChAT. A count of random cells was not performed for this reason.

The extent of loading with 5-HT was variable. In some preparations a large number of 5-HT-immunoreactive varicosities could be observed in the ganglia of the myenteric plexus, and immunoreactive cell bodies could be identified. In other preparations relatively few varicosities or cell bodies were visible. It is unlikely that all 5-HT-handling neurons were adequately labelled in all preparations. However, 51±10% (range=31–73%) of the cells within TH-IR pericellular baskets were immunoreactive for 5-HT (Fig. 11). This was significantly different to randomly selected cells ($P<0.05$), where 4±2% were 5-HT-immunoreactive.

Discussion

Tracing technique

In this study, we have developed a method for anterograde tracing in vitro of visceral sensory and autonomic efferent nerve fibres supplying the guinea-pig intestine, and have revealed the branching patterns of the classes of nerve fibres known to be present in mesenteric nerves. These include postganglionic sympathetic efferent axons (Furness et al. 1979), and the peripheral endings of some extrinsic spinal afferent fibres (Gibbins et al. 1985). In addition, we visualised unidentified endings that may belong to vagal afferent fibres (Berthoud et al .1997) or vagal efferent fibres (Berthoud et al. 1991). In addition, intestinofugal nerve cell bodies were retrogradely labelled very effectively (Dalsgaard and Elfvin 1982; Kuramoto and Furness 1989). The method can be successfully combined with immunohistochemical localisation of other markers. There is no evidence that the technique shows any selectivity for labelling particular classes of nerve fibres and thus represents a useful addition to existing methods for studying neuronal connectivity.

For the success of this method, two main factors had to be taken into consideration: firstly axons begin to degenerate after they are severed from their cell bodies, with a time course similar to the slow axonal transport (Williams and Hall 1971; Watson 1976; Cancalon 1983). Secondly, filling of nerve fibres by axonal transport takes time. A prerequisite was to identify a combination of conditions in which these two constraints could be reconciled. The key to success was the development of a medium in which to apply the tracers and the choice of the tracer itself. The composition of the medium which worked best is similar to that of the intracellular space, with physiological cation concentra-

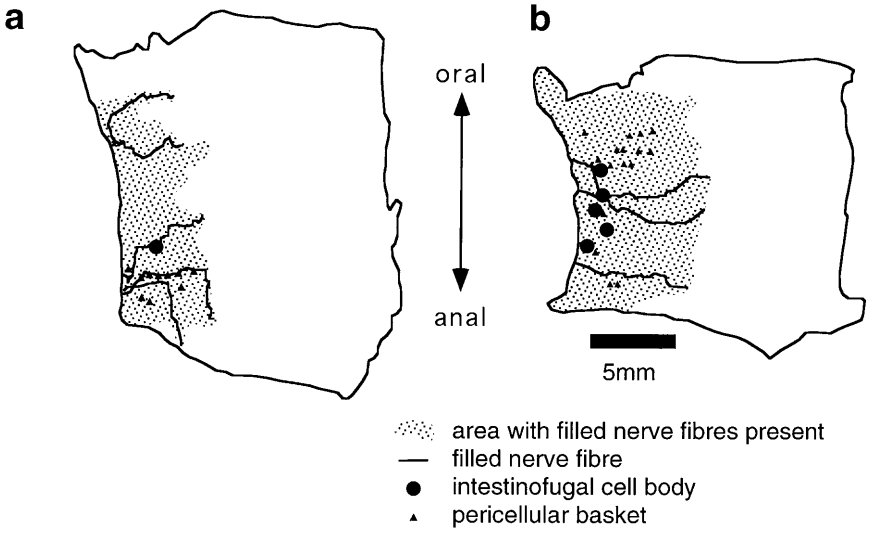

Fig. 7 Plots of the distribution of nerve fibres anterogradely labelled in two preparations from the one animal using biotinamide in tracer solutions with 3 mM calcium chloride (**a**), and without (**b**). Plots of the distribution of fibres were used to compare the distance over which nerve fibres were successfully labelled. In the preparation with calcium added to the tracer solution, the distance of filling of nerve fibres was less than in the control preparation

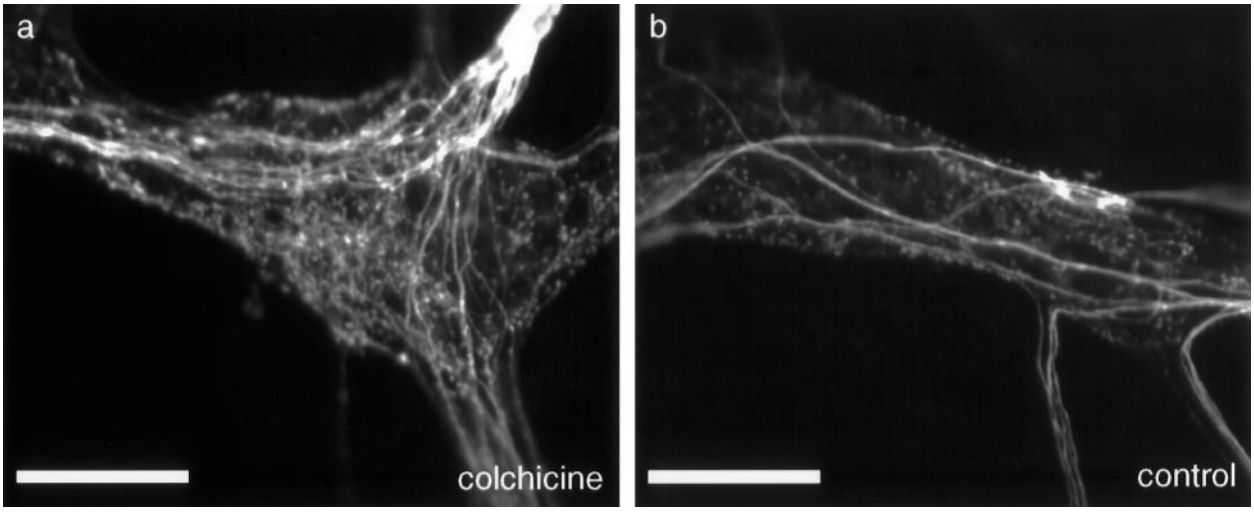

Fig. 8a,b Effect of colchicine on anterograde filling of fibres in myenteric ganglia. Following incubation with 80 μm colchicine (**a**), labelling of nerve fibres was similar to control (**b**). *Bar* 100 μm

tions and pH, with ATP as an energy source. This suggests that the intracellular-like tracer medium facilitated rapid access of the tracer into the axon.

The best tracers were the two forms of biotinamide, i.e. Neurobiotin and biotinamide hydrobromide. Biotinamide and its related compounds are small polar molecules which have been used as anterograde tracers in the central nervous system following either intracellular injection (Horikawa and Armstrong 1988; Hu et al. 1990) or extracellular application around intact nerve cell bodies (King et al. 1989; Izzo 1991; Lapper and Bolam 1991). It appears that the synthetic intracellular medium developed in this study facilitates the access of biotinamide to the axoplasm of axons. Several observations suggest that the transport of biotinamide or biocytin is by passive diffusion rather than an active process: The axons and cell bodies were evenly labelled (in contrast to granular labelling with DiI), the transport occurred at room temperature, and colchicine, which disrupts microtubules and hence fast axonal transport, did not abolish labelling. Saponin slightly improved the intensity of staining. It may do so by increasing the permeability of the cell membranes, allowing more tracer to enter the axons. Saponins are glycosides which complex with cholesterol in animal cell membranes, forming pores (Berry et al. 1991). Although they have been used extensively to permeabilise hepatocytes and other cell lines, they have not been widely used in the nervous system (but see Forman et al. 1983).

Other tracer substances have been shown to work in anterograde tracing in vivo. There are excellent studies of the vagal preganglionic parasympathetic or sensory neurons to the gastrointestinal tract using wheat-germ agglutinin-horseradish peroxidase (WGA-HRP), DiI and HRP in vivo (e.g. Clerc and Condamin 1987; Neuhuber 1987; Berthoud et al. 1991; Kressel et al. 1994; Berthoud et al. 1997). Anterograde labelling in vivo of spinal sensory neurons to the viscera is technically difficult, due to the inaccessibility of the dorsal root ganglia, but has been achieved for the

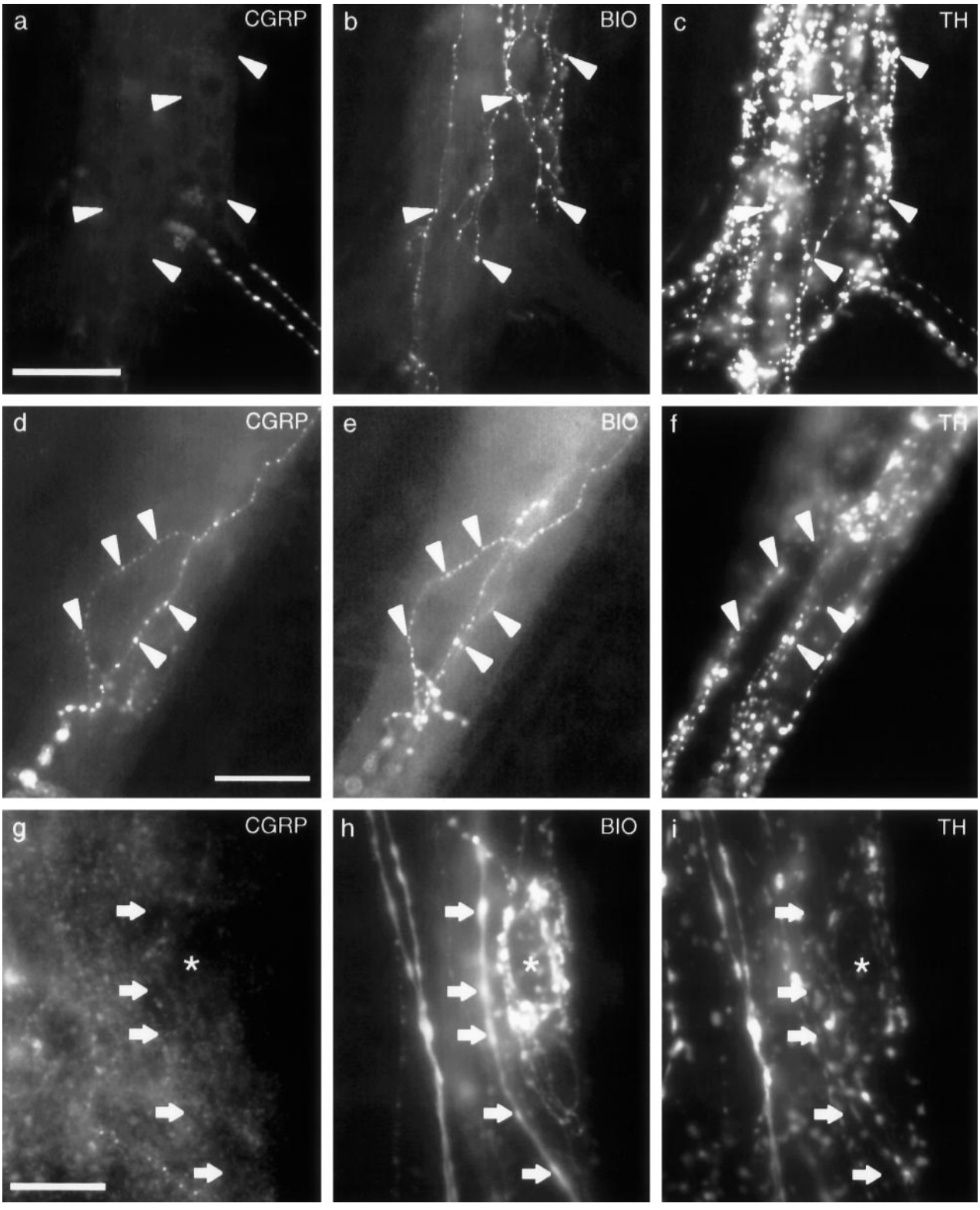

Fig. 9 Nerve fibres anterogradely filled with biotinamide (**b,e,h**) and corresponding immunofluorescence for CGRP (**a,d,g**) and TH (**c,f,i**). Most of the biotinamide-filled varicosities (*upper panel)* were immunoreactive for TH (*arrowheads*), indicating that most anterogradely labelled fibres were of postganglionic sympathetic origin. However, as non-varicose and intervaricose fibres are readily demonstrated with biotinamide immunoreactivity, the patterns of staining appear different from those shown by TH. The CGRP-immunoreactive fibres visible in this ganglion (*bottom right*, **a**), were not filled with biotinamide. In another preparation, a branching nerve fibre labelled with biotinamide (**e**) was immunoreactive for CGRP (**d**) but not TH (*arrowheads*, **f**). Some baskets of biotinamide-labelled varicosities (*asterisk*, **h**) were immunoreactive for neither CGRP (**g**) nor TH (**i**) as were the associated coarse varicose fibres (*arrows*). *Bars* 50 μm

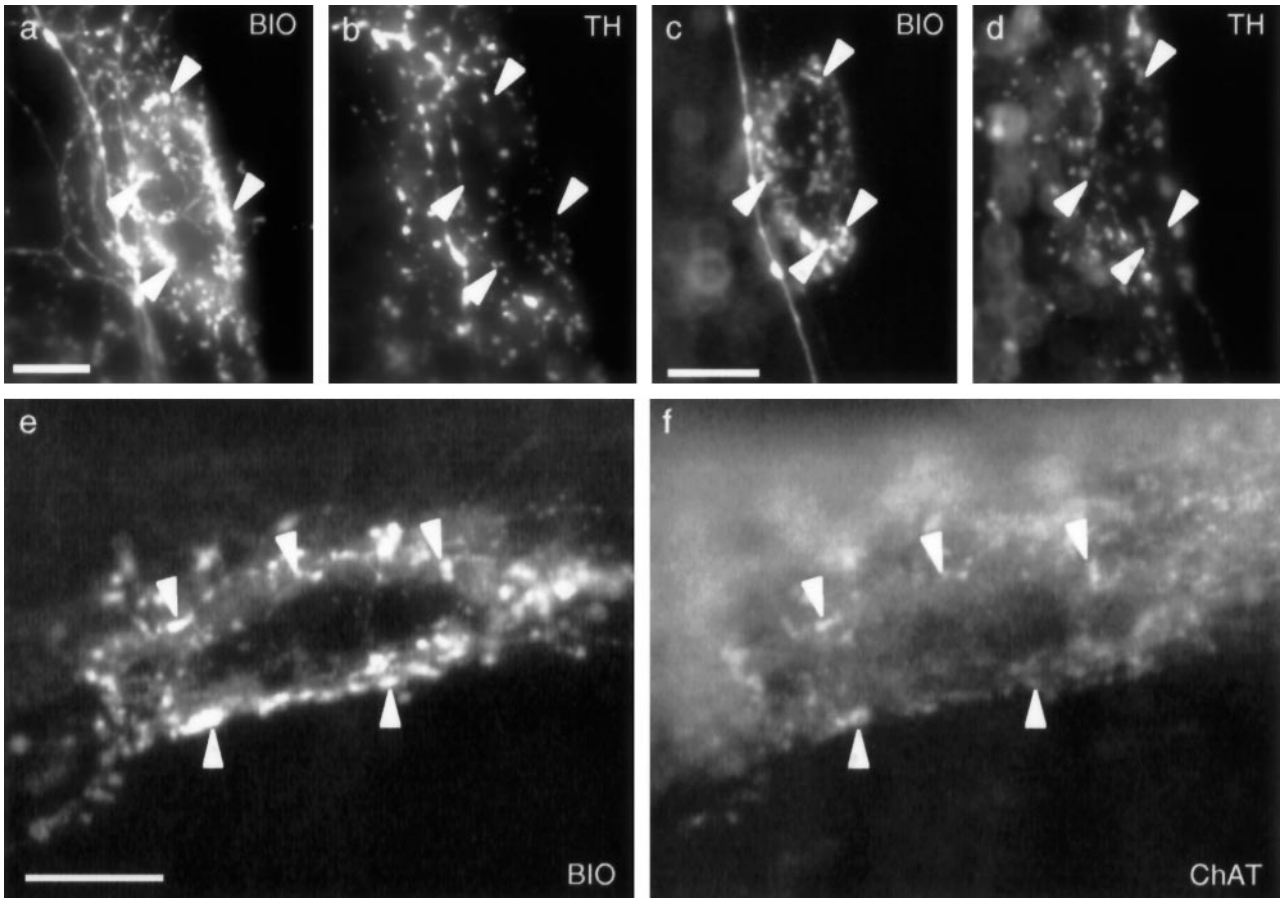

Fig. 10 Two anterogradely labelled pericellular baskets (**a,c**), consisting predominantly of varicosities which are not immunoreactive for TH (**b,d**). The coarse anterogradely filled axon in **a** is TH-immunoreactive (**b**). The coarse anterogradely filled axon in **c** is not TH-immunoreactive (**d**). A pericellular basket, anterogradely labelled with biotinamide(**e**), contained many varicosities with ChAT immunoreactivity (*arrowheads*, **f**). Typically, ChAT-immunoreactive varicosities labelled with this antiserum do not have a sharp outline, but it was clear that substantial numbers of biotinamide-labelled profiles in some baskets also contained immunofluorescence for ChAT. *Bars* 20 μm

gastro-esophageal junction (Clerc and Mazzia 1994; Mazzia and Clerc 1997), mesenteric ganglia (Aldskogius et al. 1986), pylorus (Lindh et al. 1989) and spleen (Elfvin et al. 1992). The successful filling of axons by intra-axonal injection of tracers obtained by Jacquin et al. (1992) is technically demanding and only very large central processes of some sensory neurons were randomly filled. In our hands, WGA, fast blue, fluorogold and biotinylated dextrans were not successful when applied in the same way as biotinamide.

Anterograde labelling of axons by DiI in organ culture of gut has been reported previously but occurred over several days and thus required nerve fibres to remain attached to their cell bodies (Brookes et al. 1991, 1996). Anterograde filling of fibres with biotinamide occurred more rapidly than this. In addition our method demonstrates the finest details of filled nerve varicose terminals as the confocal microscopy of filled baskets shows, unlike DiI-labelled nerve fibres.

Nature of labelled nerve fibres

This technique is compatible with simultaneous immunofluorescence techniques, and enabled the types of fibres labelled to be investigated. The majority of the filled fibres were immunoreactive for TH, with both smooth non-terminal and varicose terminal axons, forming a loose network between the cell bodies and on the surfaces of the ganglia, confirming previous results (Llewellyn-Smith et al. 1981). Our study demonstrates well the fine branching of the terminals of single TH-immunoreactive nerve fibres. These fibres are likely to be postganglionic sympathetic fibres (Furness et al. 1979; Macrae et al. 1986), although it is possible that the small classes of spinal and vagal sensory neurons which have TH immunoreactivity (Kummer et al. 1990, 1993) may also project to the intestine. In the TH-immunoreactive, biotinamide-filled axons, some varicose and non-varicose parts of the axons did not show TH immunoreactivity. This suggests that this cytoplasmic enzyme is not present in detectable amounts in all parts of the axons

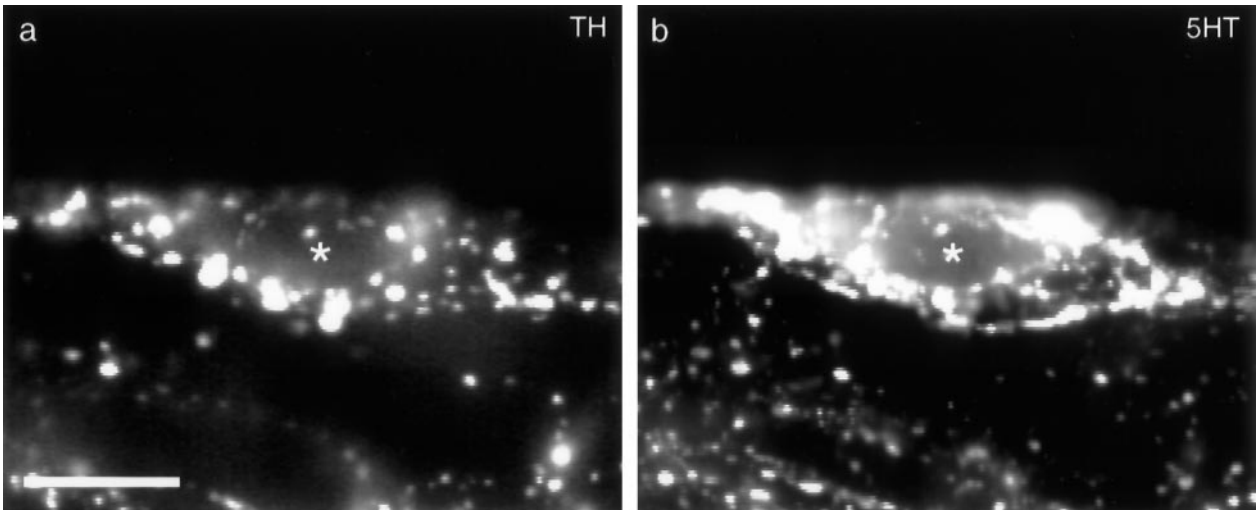

Fig. 11 TH-immunoreactive pericellular basket (*asterisk*, **a**) surrounding a cell which is immunoreactive for 5-HT (**b**). *Bar* 20 μm

and that visualisation with TH antibodies does not reveal the full extent of noradrenergic nerve terminals.

The diffuse arrangement of sympathetic terminals within myenteric ganglia is suitable for the action of sympathetic neurotransmitters on many enteric neurons throughout the ganglia. This is reflected in the electrophysiological evidence suggesting that noradrenaline acts presynaptically on the terminals of enteric neurons to inhibit the release of transmitter (Paton and Vizi 1969; Manber and Gershon 1979; Furness and Costa 1987), but does not hyperpolarise myenteric neurons directly (Nishi and North 1973; Hirst and McKirdy 1974). It is well established that one of the main actions of sympathetic nerves is to inhibit intestinal motility, by acting on myenteric neurons (Furness and Costa 1987).

TH-immunoreactive nerve fibres which form pericellular baskets around individual myenteric neurons have been described previously (Llewellyn-Smith et al. 1981), and are likely to represent specific enteric neuronal targets of sympathetic postganglionic nerve fibres. Baskets of anterogradely filled sympathetic nerve fibres were indeed observed in the present study, as demonstrated by the presence of large numbers of TH-immunoreactive varicosities in anterogradely labelled pericellular baskets. The cells within these baskets did not contain visible biotinamide fluorescence, indicating that transneuronal transport of biotinamide is not detectable under these conditions although it has been reported following intra-axonal injection (Luo and Dessem 1996). Pericellular baskets are suggestive of a strong synaptic input to the nerve cell contained within the basket. Therefore it is possible that a strong sympathetic inhibition of enteric neuronal activity may be exerted on one or more specific classes of myenteric neurons. Given that fewer than 1% of myenteric neurons are surrounded by these baskets, it is unlikely that many recordings from the myenteric neurons within these baskets have been made during random intracellular impalements.

In order to determine the classes of myenteric neuron around which the sympathetic baskets lay, double-labelling immunohistochemistry was performed, using anti-TH antibodies to identify postganglionic sympathetic nerve fibres, and neurochemical markers specific for the different classes of myenteric neuron (Costa et al. 1996). The baskets appeared to be targeting specific classes of myenteric neurons, since particular markers were present in a statistically higher or lower proportion of cells than would be expected by random sampling. The estimate of various populations, performed by random sampling, is in agreement with published results (Furness et al. 1994; Costa et al. 1996).

Most of the baskets were around 5-HT-handling neurons, which are a class of descending interneuron (Furness and Costa 1982; Meedeniya et al. 1998). Baskets were only rarely found around sensory neurons (calbindin), longitudinal muscle motor neurons or ascending interneurons (calretinin), SOM-immunoreactive descending interneurons (SOM) or secreto-/vasomotor neurons projecting to the submucosa (SOM, VIP). They were never found around NOS-immunoreactive circular muscle inhibitory motor neurons or NOS-immunoreactive descending interneurons. For approximately 40% of baskets, the myenteric neuron did not appear to contain any of the markers used. A proportion of these may be accounted for by 5-HT-handling cells, which were stained too faintly to be detected in this study. Alternatively these cells may belong to the classes which do not contain any of the specific labels used, i.e. the circular muscle excitatory motor neurons and some of the descending interneurons (Costa et al. 1996). Over 60% of the cells were seen to contain ChAT, although it is likely that this is a substantial underestimate, as very few myenteric neurons do not contain one of NOS, VIP or ChAT (Costa et al. 1996). The 5-HT-handling cells contain ChAT, as do the classes of cells for which no other specific label is known (Costa et al. 1996).

The finding that enteric 5-HT-containing neurons are specific targets of sympathetic noradrenergic nerve fibres in the myenteric plexus supports previous work by other in-

Table 2 Proportion of TH baskets surrounding cells containing the different immunohistochemical markers used

Neuronal marker	Proportion in TH baskets	Proportion of total cells, by random sampling	Statistically significant difference
5-HT	51±10%	4±2%	[a]
Calbindin	3±1%	23±2%	[a]
SOM	3±1%	4±1%	
Calretinin	2±1%	24±1%	[a]
VIP[a]	0%	1±1%	
NOS	0%	30±3%	[a]
ChAT	61±5%	N/A	N/A

[a] In preparations without colchicine treatment, only secretomotor myenteric neurons show VIP immunoreactivity (Costa et al. 1996)

vestigators. Noradrenergic varicosities form axo-axonic synaptic specialisations with 5-HT-containing neurons (Gershon and Sherman 1982; Gershon and Sherman 1987). Furthermore, many 5-HT-containing neurons are contacted by noradrenergic varicosities, whereas non-5-HT-handling neurons rarely are (Gershon and Sherman 1987). There is also evidence that α_2-adrenergic receptor stimulation inhibits the release of 5-HT from myenteric neurons (Wood and Mayer 1979; Gershon 1981; Gershon and Sherman 1982), whereas β-adrenergic receptor stimulation facilitates its release (Gershon 1981; Gershon and Sherman 1982). This suggests that sympathetic inhibition of enteric neural activity occurs in a diffuse manner to mainly inhibit excitatory myenteric motor pathways, and in a target-specific manner to inhibit a class of descending interneurons, the role of which remains to be determined.

Surprisingly, we found a substantial number of baskets containing anterogradely filled nerve fibres which were not immunoreactive for TH. This specialised pericellular arrangement has not been described before. Many of these nerve fibres contained ChAT, and none of them contained CGRP immunoreactivity. These nerve fibres run within the mesenteric nerves and may represent axons of preganglionic parasympathetic cholinergic neurons (Berthoud et al. 1991), postganglionic cholinergic sympathetic neurons, axon collaterals of the intestinofugal neurons (a proportion of which are likely to be cholinergic; Szurszewski and King 1989; Mann et al. 1995; current results), or axons of extrinsic sensory neurons. Not all spinal sensory neurons supplying the intestine contain CGRP (Dockray et al. 1991), and there is evidence that a proportion of both spinal and vagal sensory neurons may contain ChAT (Palouzier et al. 1987; Ternaux et al. 1989; Palouzier Paulignan et al. 1991; Sann et al. 1995).

Efferent vagal parasympathetic fibres form baskets around enteric neurons in the stomach (Kirchgessner and Gershon 1989; Berthoud and Powley 1992; Berthoud 1995). In addition vagal afferent fibres have been shown to give rise to intraganglionic lamellar endings (IGLEs) throughout the gastrointestinal tract of the rat (Berthoud et al 1997), although these are rather less dense in the ileum than further proximally. The pericellular baskets revealed in the present study in the ileum were considerably less extensive than IGLEs and appeared rather more varicose and thus may not be of vagal afferent origin. The origin of the nerve fibres forming the ChAT-immunoreactive pericellular baskets in the small intestine remains to be determined. It is interesting that both TH- and ChAT-immunoreactive nerve fibres often form pericellular baskets around the same myenteric nerve cell bodies. This suggests that there is a convergence of highly specific input from both sympathetic and a yet-to-be-determined cholinergic source.

Our work also revealed well the other expected class of nerve fibres known to run within the mesenteric nerves: i.e. the extrinsic spinal sensory neurons, characterised by CGRP immunoreactivity. These are known to be sparser than the sympathetic nerve fibres in the myenteric plexus (Gibbins et al. 1985). Indeed, relatively few filled fibres were labelled with CGRP in our experiments and we confirmed that they do not appear to form any specialised arrangement around myenteric neurons, at least at the light-microscopic level.

Intestinofugal neurons

A number of nerve cell bodies were filled with biotinamide in myenteric ganglia in each preparation. These retrogradely filled neurons represent the intestinofugal neurons with their axons running within the mesenteric nerves to the prevertebral ganglia, where they synapse with some of the classes of sympathetic postganglionic neurons (Dalsgaard and Elfvin 1982; Kuramoto and Furness 1989). The detailed morphology of these neurons is revealed extremely well by this method, as demonstrated by confocal microscopy. The nerve cell bodies were small, and unipolar, with a variable number or lamellar or short filamentous dendrites. Thus this method is also highly suitable for retrograde tracing from peripheral nerve trunks in vitro. There is pharmacological evidence that these neurons, in addition to containing a number of other neurochemicals, utilise acetylcholine as a major neurotransmitter, and could therefore be expected to contain ChAT, as has been demonstrated previously (Mann et al. 1995). Indeed, we found that a number of retrogradely labelled nerve cell bodies contained ChAT, although the proportion of cells is likely to be an underestimate, as staining for ChAT was optimised for visualisation of nerve terminals, and hence the staining of cell bodies was compromised (Steele et al. 1991).

In conclusion, the technique presented here should be of use in tracing neuronal pathways in a variety of tissues which can be maintained for short periods in organ culture and which have readily accessible nerve trunks.

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